MOLECULAR AND CELLULAR BIOLOGY, July 1995, p. 3714–3721 Vol. 15, No. 7
0270-7306/95/$04.00+0

Dioxin Induces Localized, Graded Changes in Chromatin Structure: Implications for *Cyp1A1* Gene Transcription

STEVEN T. OKINO AND JAMES P. WHITLOCK, JR.*

Department of Molecular Pharmacology, Stanford University School of Medicine, Stanford, California 94305-5332

Received 8 March 1995/Returned for modification 4 April 1995/Accepted 12 April 1995

In mouse hepatoma cells, the environmental contaminant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, or dioxin) induces *Cyp1A1* gene transcription, a process that requires two basic helix-loop-helix regulatory proteins, the aromatic hydrocarbon receptor (AhR) and the aromatic hydrocarbon receptor nuclear translocator (Arnt). We have used a ligation-mediated PCR technique to analyze dioxin-induced changes in protein-DNA interactions and chromatin structure of the *Cyp1A1* enhancer-promoter in its native chromosomal setting. Dioxin-induced binding of the AhR/Arnt heteromer to enhancer chromatin is associated with a localized (about 200 bp) alteration in chromatin structure that is manifested by increased accessibility of the DNA; these changes probably reflect direct disruption of a nucleosome by AhR/Arnt. Dioxin induces analogous AhR/Arnt-dependent changes in chromatin structure and accessibility at the *Cyp1A1* promoter. However, the changes at the promoter must occur by a different, more indirect mechanism, because they are induced from a distance and do not reflect a local effect of AhR/Arnt binding. Dose-response experiments indicate that the changes in chromatin structure at the enhancer and promoter are graded and mirror the graded induction of *Cyp1A1* transcription by dioxin. We discuss these results in terms of a TCDD-induced shift in an equilibrium between nucleosomal and nonnucleosomal chromatin configurations.

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD, or dioxin) is the prototype for a class of halogenated aromatic hydrocarbons that are widespread environmental contaminants. Such compounds elicit a variety of biochemical, immunological, reproductive, and neoplastic effects in animals and pose a potential risk to human health (2, 42, 46). The diversity of TCDD's effects may reflect its ability to alter gene expression in a species- and tissue-specific fashion (37, 42, 53).

The aromatic hydrocarbon receptor (AhR) is an intracellular protein that mediates the biological responses to TCDD. The AhR contains a basic helix-loop-helix (bHLH) motif and acts in partnership with a second bHLH protein, the Ah receptor nuclear translocator (Arnt). The AhR/Arnt heteromer functions as a ligand-dependent DNA-binding transcription factor in inducing the expression of dioxin-responsive genes (4, 9, 19, 30, 43, 44, 47, 51–53).

The TCDD-inducible *Cyp1A1* gene in mouse hepatoma cells is a useful experimental system for analyzing the mechanism of dioxin action. Induction is rapid, direct, and AhR and Arnt dependent and occurs at the level of transcription (13, 15, 37, 53). The DNA upstream of the *Cyp1A1* gene contains a dioxin-responsive enhancer, which has multiple binding sites for AhR/Arnt and is located several hundred base pairs upstream of the transcriptional promoter. The promoter has binding sites for several constitutively expressed general transcription factors but does not bind AhR/Arnt. The enhancer and promoter function in concert during the transcriptional response to TCDD (53).

Changes in chromatin structure accompany the induction of *Cyp1A1* transcription by TCDD. When the gene is inactive, the regulatory region assumes a nucleosomal configuration, and there is no detectable binding of proteins to either the enhancer or the promoter in intact cells. Exposure of cells to TCDD leads rapidly to the binding of AhR/Arnt to the enhancer; this protein-DNA interaction is associated with the disruption of the nucleosomal organization of the regulatory region and with the binding of general transcription factors to the promoter (7, 34, 54, 55). These observations raise questions about the mechanism by which events at the enhancer influence the function of the promoter, which is located hundreds of base pairs downstream, and the role of chromatin structure in this process.

Here, we have analyzed in greater detail the alterations in chromatin structure that accompany the induction of *Cyp1A1* transcription by TCDD. We have focused on the enhancer-promoter region, and we have used a ligation-mediated PCR (LMPCR) technique that allows us to analyze the region in its native chromosomal setting. We find that TCDD induces localized and discontinuous changes in chromatin structure and that these changes display a graded response to the inducer. Our findings imply that AhR/Arnt disrupts chromatin structure by different mechanisms at the enhancer and the promoter. To account for these observations, we envision that TCDD induces a shift in an equilibrium between nucleosomal and nonnucleosomal chromatin structures.

MATERIALS AND METHODS

Materials. DNase I and micrococcal nuclease were purchased from Boehringer Mannheim. T4 DNA ligase was purchased from Promega. T4 polynucleotide kinase, *Mnl*I, and Vent DNA polymerase were purchased from New England Biolabs. All other molecular biological reagents were from Sigma or Bethesda Research Laboratories. [γ-^{32}P]ATP (3,000 Ci/mmol; 1 Ci = 37 MBq) was from Amersham. TCDD was obtained from the National Cancer Institute Chemical Carcinogen Reference Standard Repository.

Cell culture. Wild-type (Hepa 1c1c7) and Arnt-defective (BP^{r}c1) mouse hepatoma cells were grown to 80% confluence on 100-mm tissue culture plates (about 10^7 cells per plate) and treated with TCDD (dissolved in dimethyl sulfoxide) for 2 h. In some experiments cells were treated with actinomycin D (2 μg/ml), added 30 minutes before TCDD.

Studies with DMS and LMPCR. Experiments with dimethyl sulfate (DMS) and LMPCR to analyze protein-DNA interactions in intact cells were performed as previously described (54, 55).

* Corresponding author. Phone: (415) 723-8233. Fax: (415) 725-2952.

Preparation of nuclei. For the isolation of nuclei, all buffers were kept on ice and the cells were kept ice-cold unless otherwise noted. Four plates of cells (about 4×10^7 cells) were harvested per sample. The culture medium was removed, and the cells were rinsed with 5 ml of buffer A (0.3 M sucrose, 60 mM KCl, 60 mM Tris-HCl [pH 8.0], 2 mM EDTA). Then 3 ml of buffer A was added to each plate, and the cells were collected in a 15-ml conical centrifuge tube by scraping with a rubber policeman. The cells were then pelleted by centrifugation at $500 \times g$ for 5 min at 4°C and resuspended in 4 ml of buffer A. Then 4 ml of buffer A and 0.5% Nonidet P-40 were added, and the cells were left on ice for 5 min. The nuclei were then pelleted by centrifugation at $500 \times g$ for 5 min at 4°C.

DNase I digestion. Nuclei were resuspended in 1 ml of buffer B (150 mM sucrose, 80 mM KCl, 35 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.4], 5 mM K_2HPO_4, 5 mM $MgCl_2$, 2 mM $CaCl_2$) at 22°C. Then 1 ml of buffer B plus 10 μg of DNase I per ml was added, and the nuclei were digested for 90 s at 22°C. Buffer C (2 ml; 20 mM Tris-HCl [pH 8.0], 20 mM NaCl, 20 mM EDTA, 1% sodium dodecyl sulfate [SDS], 600 μg of proteinase K per ml) was then added to stop the reaction. The viscous solution was then incubated at 37°C for 3 h.

***Mnl*I digestion.** Nuclei were resuspended in 200 μl of NEBuffer 2 (New England Biolabs) at 30°C. Then 200 μl of NEBuffer 2 plus 200 U of *Mnl*I per ml was added, and the nuclei were digested for 30 min at 30°C. Buffer C (400 μl) was then added to stop the reaction. The viscous solution was then incubated at 37°C for 3 h.

Micrococcal nuclease digestion. Nuclei were resuspended in 1 ml of buffer D (150 mM sucrose, 50 mM Tris-HCl [pH 7.5], 50 mM KCl, 1 mM $CaCl_2$) at 37°C. Buffer D (1 ml) plus 5 U of micrococcal nuclease per ml was then added, and the nuclei were digested for 5 min at 37°C. Buffer C (2 ml) was then added to stop the reaction. The viscous solution was then incubated at 37°C for 3 h.

Isolation of DNA. The solubilized nuclei were extracted once with an equal volume of phenol and once with phenol-chloroform (1:1). The nucleic acid was then precipitated from the aqueous phase with ethanol and pelleted by centrifugation. The pellet was resuspended in 360 μl of water. Then 40 μl of 10× RNase buffer (500 mM Tris-HCl [pH 7.5], 100 mM $MgCl_2$, 50 mM dithiothreitol) and 5 μl of 10-mg/ml RNase A were added, and the solution was digested at 37°C for 1 h. The viscous solution was extracted with an equal volume of phenol-chloroform, and the DNA was precipitated with ethanol. The DNA was pelleted by centrifugation and resuspended in 200 μl of water, and the concentration of DNA was determined by spectrophotometry. The DNA was diluted with water to a final concentration of 1 mg/ml.

LMPCR analysis of DNA digested with DNase I or *Mnl*I. DNA was analyzed by LMPCR as previously described (14, 35, 48) with the following primer sets. Set A consisted of the following: primer 1, 5′-CTTACACTAATCTCACTCT GGGAG-3′; primer 2, 5′-GGCCAGAGAGCACCTGCAAAACAGC-3′; and primer 3, 5′-AGCACCTGCAAAACAGCCAGCTAGGCGTG-3′. Set B consisted of the following: primer 1, 5′-TTGTCGCGCCTTGCAAAGCATA GAT-3′; primer 2, 5′-AAACCCACCCAACGCCAGGAGAGCT-3′; and primer 3, 5′-CCCAACGCCAGGAGAGCTGGCCCTTTA-3′. Set C consisted of the following: primer 1, 5′-ATCCATCCCCACCCTCTAGATGAAG-3′; primer 2, 5′-CGAACTTCGGCCGATACCCAATTTG-3′; and primer 3, 5′-GGCCGAT ACCCAATTTGTGGGGCACAG-3′. Set D consisted of the following: primer 1, 5′-CCTCAGTGGGATTATGCACTGT-3′; primer 2, 5′-CTGTCCATGGAG GACCTTGAAAGTG-3′; and primer 3, 5′-CTGCCTAGAGCACTCCCTAAG GCTGTCC-3′.

For all primer sets, the annealing temperatures were as follows: primer 1, 47°C; primer 2, 60°C; and primer 3, 66°C. All samples were denatured for 3 min at 95°C immediately prior to the first PCR cycle. The conditions for 15 cycles of amplification were 1 min at 94°C for denaturation, 2 min at 60°C for annealing primer 2, and 3 min at 76°C for extension. The time period of the extension cycle was increased an additional 5 s for every cycle. The conditions for primer extension of the end-labeled primer 3 were 3 min at 95°C for denaturation, 2 min at 66°C for annealing primer 3, and 10 min at 76°C for extension. For studies of accessibility, equal amounts of the LMPCR mixture (representing equal amounts of genomic DNA) were analyzed on an 8% denaturing polyacrylamide gel. The gel was then fixed, dried, and autoradiographed with Amersham Hyperfilm-MP. For studies of protein-DNA interactions (DNA footprinting), some LMPCR samples were diluted to equalize banding intensity as visualized by autoradiography.

LMPCR analysis of DNA digested with micrococcal nuclease. To map double-stranded micrococcal nuclease digestion sites, the isolated DNA was phosphorylated with polynucleotide kinase and the first primer extension step was omitted, as previously described (32). Otherwise, LMPCR was done as described above.

RESULTS

DNase I versus DMS for analyzing protein-DNA interactions in situ. Previously, we analyzed the TCDD-induced binding of AhR/Arnt and other transcription factors to the *Cyp1A1* regulatory region in intact cells with DMS as a DNA-reactive probe. Such experiments provide valuable information about the pattern of protein binding to the enhancer and promoter in their native chromosomal context (54, 55). However, DMS is relatively insensitive to histone-DNA interactions; even in the nucleosome, DMS readily modifies DNA (31). Therefore, DMS is not optimal for analyzing the changes in chromatin structure that accompany the induction of *Cyp1A1* transcription by TCDD. In contrast, nucleases have diminished access to DNA in chromatin and are likely be more useful for studying TCDD-induced changes in chromatin structure (16). To test this idea, we compared the effectiveness of DMS and DNase I in detecting (i) the binding of AhR/Arnt to chromatin and (ii) an altered chromatin structure associated with AhR/Arnt binding. Our findings indicate that both DMS and DNase I detect the TCDD-inducible binding of AhR/Arnt to its recognition sequence (Fig. 1; compare lanes 2 and 3 and lanes 5 and 6); however, only DNase I detects an associated increase in reactivity of the DNA that flanks the AhR/Arnt binding site (Fig. 1; compare lanes 5 and 6). The increased susceptibility to nuclease digestion likely reflects a change in chromatin structure (such as loss of a nucleosomal configuration). Therefore, we used nuclease digestion combined with an LMPCR technique to analyze changes in protein-DNA interactions and nuclease susceptibility that accompany the induction of *Cyp1A1* transcription by TCDD.

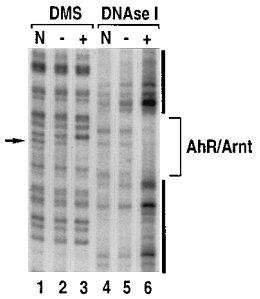

FIG. 1. DMS and DNase I as probes of the *Cyp1A1* regulatory region. Untreated (−) and TCDD-treated (1 nM, 2 h) (+) Hepa 1c1c7 cells were exposed to DMS or used to isolate nuclei, which were subsequently exposed to DNase I. Naked genomic DNA (N) was also exposed to these agents in vitro. The pattern and extent of DNA modification were assessed by LMPCR with primer set B. The arrow indicates a guanine residue protected from DMS modification in response to TCDD. The vertical lines indicate regions which exhibit a TCDD-induced increase in DNase I susceptibility. The binding site for AhR/Arnt is indicated.

TCDD-induced changes in transcription factor binding to the *Cyp1A1* regulatory region. Our studies involve the preparation of nuclei prior to nuclease digestion. To verify that the experimental manipulations do not generate artifacts in the binding of transcription factors to the *Cyp1A1* regulatory region, we analyzed the pattern of TCDD-inducible protein-DNA interactions at the enhancer and promoter. The data presented in Fig. 2 are representative examples of our findings. In uninduced cells, the enhancer-promoter region exhibits no areas of nuclease resistance, a finding which implies the absence of DNA-bound regulatory proteins. This observation is consistent with our previous results with DMS (54, 55) and argues against the idea that a repressor protein(s) inhibits *Cyp1A1* transcription in uninduced cells. Our data also imply that constitutively expressed general transcription factors are unable to bind to the enhancer-promoter region in uninduced cells. The nucleosomal organization of the DNA plausibly accounts for this result (34).

Exposure of cells to TCDD is associated with the appearance of multiple nuclease-resistant regions, implying the presence of DNA-bound proteins. At the enhancer, nuclease resistance appears at several binding sites for AhR/Arnt; in

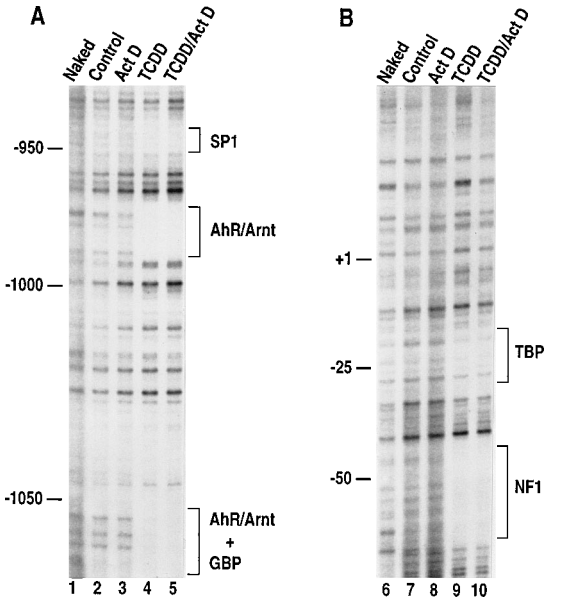

FIG. 2. Analysis of protein-DNA interactions at the *Cyp1A1* regulatory region with DNase I and LMPCR. Nuclei isolated from untreated Hepa 1c1c7 cells (control) or cells treated with TCDD (1 nM, 2 h) and/or actinomycin D (2 μg/ml, 2.5 h) (Act D) were exposed to DNase I, and the cleavage pattern was analyzed by LMPCR. Naked genomic DNA digested with DNase I in vitro was also analyzed. Distances (in base pairs) from the *Cyp1A1* transcriptional start site are indicated. (A) Protein-DNA interactions on the *Cyp1A1* enhancer; LMPCR analysis with primer set B. Binding sites for AhR/Arnt, Sp1, and the G-box-binding protein (GBP) are indicated. The samples loaded in lanes 4 and 5 were diluted eightfold to normalize banding intensity. (B) Protein-DNA interactions on the *Cyp1A1* promoter; LMPCR analysis with primer set D. Binding sites for nuclear factor 1 (NF1) and TBP are indicated. The samples loaded in lanes 9 and 10 were diluted 12-fold to normalize banding intensity.

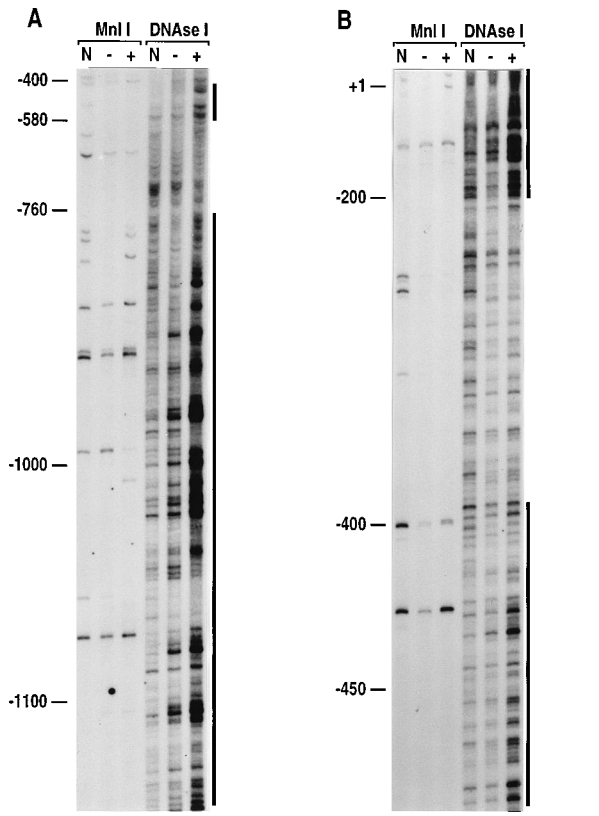

FIG. 3. TCDD induces localized increases in accessibility of the *Cyp1A1* regulatory region. Nuclei isolated from untreated Hepa 1c1c7 cells (−) or cells treated with TCDD (1 nM, 2 h) (+) were exposed to DNase I or *Mnl*I, and the cleavage pattern was analyzed by LMPCR. Naked genomic DNA digested with these agents in vitro was also analyzed (N). All bands in the *Mnl*I-digested samples represent bona fide *Mnl*I cleavage sites, as predicted by DNA sequence analysis. Distances (in base pairs) from the *Cyp1A1* transcriptional start site are indicated. The vertical lines indicate regions which exhibit a TCDD-induced change in accessibility. (A) LMPCR analysis with primer set A. (B) LMPCR analysis with primer set C.

addition, we observe TCDD-inducible nuclease resistance at a recognition motif for Sp1 and at a G-rich sequence (Fig. 2A). At the promoter, TCDD induces nuclease resistance at a TATAAA sequence, at a recognition motif for the transcription factor NF1, and at a G-rich sequence (Fig. 2B and data not shown). We infer that these changes reflect the binding of the cognate proteins to DNA. Thus, our findings indicate that the binding of AhR/Arnt to the enhancer is associated with the binding of constitutively expressed transcription factors to both the enhancer and the promoter. Studies described later address the mechanism of this process in greater detail.

Our experiments with nuclei and DNase I detect each of the protein-DNA interactions found previously in studies with intact cells and DMS (54, 55). Our studies also reveal a protein-DNA interaction that is not detectable with intact cells and DMS, namely, the TCDD-inducible occupancy of the TATA box (Fig. 2B). Presumably, this reflects the binding of the TATA-binding protein (TBP) to the *Cyp1A1* promoter. Our findings imply that the isolation of nuclei and the use of nucleases do not substantially perturb protein-DNA interactions at the *Cyp1A1* enhancer and promoter. Therefore, we infer that our observations accurately reflect the behavior of the *Cyp1A1* regulatory region in its native chromosomal setting.

Actinomycin D, at a concentration that blocks *Cyp1A1* transcription by >95%, fails to inhibit the TCDD-induced binding of transcription factors to the *Cyp1A1* regulatory region (Fig. 2A and B). Therefore, the protein-DNA interactions reflect primary effects of TCDD rather than responses that occur secondary to transcription. Time course studies indicate that the TCDD-induced changes occur within 30 to 60 min (data not shown), which is the same time frame as induction of *Cyp1A1* transcription by TCDD (21). These observations tend to implicate protein-DNA interactions in the induction mechanism.

TCDD induces localized increases in nuclease susceptibility at the *Cyp1A1* regulatory region. The binding of transcription factors to *Cyp1A1* chromatin represents one type of alteration induced by TCDD. In addition, we find that the chromatin in the vicinity of the protein binding sites exhibits an increase in nuclease susceptibility, a result that presumably reflects an alteration in chromatin structure. Interestingly, the increased nuclease susceptibility is not uniform but is localized to discrete domains of chromatin. For example, the region that extends from about −580 to −400 exhibits a TCDD-induced increase in susceptibility to DNase I and to the restriction enzyme *Mnl*I (Fig. 3A and B). This region is centered around a binding site for AhR/Arnt; there is no evidence that other proteins bind to DNA within this region. Therefore, we infer that the binding of AhR/Arnt to the enhancer alters the structure of about 180 bp of DNA.

In contrast, the neighboring regions of chromatin, both upstream (from about −580 to −760) and downstream (from about −400 to −200), exhibit no increases in nuclease susceptibility in response to TCDD (Fig. 3A and B, respectively).

Furthermore, both regions are relatively resistant to nuclease digestion compared with naked DNA. Both regions are approximately nucleosomal in size and display no evidence of transcription factor binding, even though the upstream region contains a recognition motif for Sp1. We infer, therefore, that both of these regions maintain a nucleosomal structure, even in the presence of TCDD. The absence of a 10-base DNase I ladder implies that the nucleosomes do not have a defined helical setting within these regions.

Still farther upstream, we observe a relatively long chromatin domain (spanning approximately −760 to −1600) that exhibits a TCDD-inducible increase in nuclease susceptibility (Fig. 3A and data not shown). This region contains multiple recognition motifs for AhR/Arnt, as well as an Sp1 binding site and a G-rich sequence, all of which exhibit TCDD-inducible protein binding (Fig. 2A and data not shown). Therefore, in this region of the enhancer, the data again reveal an association between the binding of AhR/Arnt and an increased susceptibility of the neighboring DNA to nucleases. Note that the decreased susceptibility of one *Mnl*I site (near bp −1000) in TCDD-induced cells occurs at an AhR/Arnt binding site; thus, that particular site is probably protected from *Mnl*I digestion by the binding of AhR/Arnt. Our results are consistent with the idea that the binding of AhR/Arnt alters the structure of enhancer chromatin and stabilizes a configuration in which the DNA is more accessible to other DNA-binding proteins. For example, even though the cell expresses Sp1 constitutively, the binding of Sp1 to the enhancer is TCDD dependent and occurs only in a nuclease-sensitive region. This behavior suggests that Sp1 binding occurs secondarily to an alteration in chromatin structure that increases the accessibility of enhancer DNA.

A third area that undergoes a TCDD-induced increase in nuclease susceptibility is localized between about −200 and +20 and includes the *Cyp1A1* promoter (Fig. 3B). The increased susceptibility is associated with the binding of several constitutively expressed general transcription factors to the promoter (Fig. 2B and data not shown). Again, these findings reveal an association between increased susceptibility to nucleases and increased binding of transcription factors. However, the promoter contains no binding sites for AhR/Arnt; therefore, the mechanism by which the changes occur at the promoter must differ from that at the enhancer.

We used micrococcal nuclease to further analyze the nuclease-resistant regions of the *Cyp1A1* regulatory region. Our findings (Fig. 4) reveal that, in both uninduced and TCDD-induced cells, the region spanning −400 to −200 is resistant to digestion, compared with naked DNA. This finding confirms our observations with DNase I and *Mnl*I and implies that the region maintains a nucleosomal configuration even in induced cells. The nuclease-resistant region is flanked by DNA that is more susceptible to micrococcal nuclease; this may be linker DNA or DNA that is in a nonnucleosomal configuration. A technical issue (lack of sufficient micrococcal nuclease cleavage sites) prevented us from analyzing the region between −760 and −580 in a similar fashion (data not shown).

Its inducibility by TCDD implies that the increased nuclease susceptibility of the *Cyp1A1* regulatory region requires AhR. The data in Fig. 5 indicate that the increase in nuclease susceptibility at the promoter also requires the Arnt protein, because it does not occur in Arnt-defective cells. Similarly, TCDD fails to elicit increased nuclease susceptibility at the enhancer in Arnt-defective cells (data not shown). Therefore, our results indicate that the TCDD-inducible changes in protein binding and nuclease susceptibility at the promoter are AhR/Arnt dependent. However, because the promoter contains no AhR/Arnt binding sites, the changes must occur by a mechanism that does not involve the binding of AhR/Arnt in the immediate vicinity. In addition, between the promoter and the nearest AhR/Arnt binding site there is a region of chromatin (spanning about −200 to −400) that does not undergo a structural change in response to TCDD. These data indicate that the TCDD-induced increase in promoter accessibility is not propagated directly along the chromatin fiber from an AhR/Arnt binding site on the enhancer. Instead, AhR/Arnt acts at a distance to alter chromatin structure at the promoter.

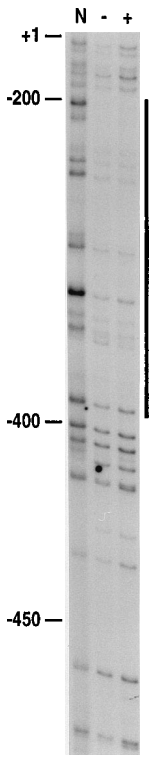

FIG. 4. Nucleosome exists between the *Cyp1A1* enhancer and promoter. Nuclei isolated from untreated Hepa 1c1c7 cells (−) or cells treated with TCDD (1 nM, 2 h) (+) were exposed to micrococcal nuclease, and the cleavage pattern was analyzed by LMPCR with primer set C. Naked genomic DNA digested with micrococcal nuclease in vitro was also analyzed (N). Distances (in base pairs) from the *Cyp1A1* transcription start site are indicated. The vertical line indicates the chromatin region resistant to micrococcal nuclease digestion.

Our findings (Fig. 5) also reveal that in Arnt-defective cells, the *Cyp1A1* promoter is less accessible than in uninduced wild-type cells; in turn, the uninduced wild-type promoter is less accessible than the promoter in TCDD-induced wild-type cells. These differences in promoter accessibility mirror the corresponding transcription rate of the *Cyp1A1* gene, which is undetectable in Arnt-defective cells, low in uninduced wild-type cells (due to weak inducers in the culture medium), and high in TCDD-induced wild-type cells. To further document the occurrence of graded changes in chromatin structure, we analyzed the nuclease susceptibility of the regulatory region as a function of TCDD concentration (Fig. 6). Our findings indicate that, at both the enhancer and the promoter, nuclease susceptibility increases as the dose of TCDD is raised; the half-maximal effect occurs between 10 and 100 pM TCDD at both regulatory elements. This concentration-effect relationship mirrors that for the induction of *Cyp1A1* transcription by TCDD (20). These findings imply that the changes at the enhancer, the changes at the promoter, and the increased transcription rate have a common mechanism, which permits a

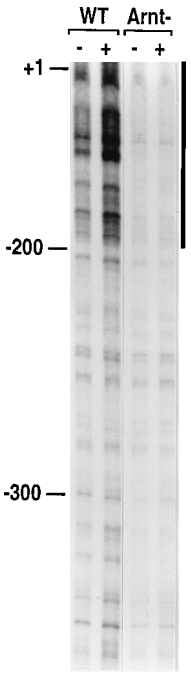

FIG. 5. TCDD-induced accessibility changes on the *Cyp1A1* promoter are Arnt dependent. Nuclei isolated from untreated (−) or TCDD-treated (1 nM, 2 h) (+) wild-type (WT) or Arnt-defective (Arnt-) Hepa 1c1c7 cells were exposed to DNase I, and the cleavage pattern was analyzed by LMPCR with primer set C. Distances (in base pairs) from the *Cyp1A1* transcription start site are indicated. The vertical line indicates the region which exhibits a TCDD-induced change in accessibility.

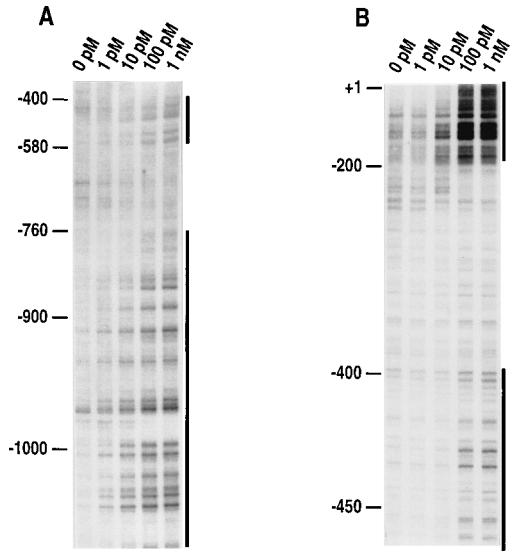

FIG. 6. TCDD induces a graded change in accessibility of the *Cyp1A1* regulatory region. Nuclei isolated from Hepa 1c1c7 cells exposed for 2 h to the indicated concentration of TCDD were exposed to DNase I, and the cleavage pattern was analyzed by LMPCR. Distances (in base pairs) from the *Cyp1A1* transcriptional start site are indicated. The vertical lines indicate regions which exhibit a TCDD-induced change in accessibility. (A) LMPCR analysis with primer set A. (B) LMPCR analysis with primer set C.

graded response to TCDD. Figure 7 summarizes the protein-DNA interactions and the regions of increased accessibility that TCDD induces in the *Cyp1A1* regulatory region. A mechanistic explanation of the transcriptional response to TCDD must account for these observations.

DISCUSSION

Cytochrome P4501A1 is an inducible microsomal enzyme that catalyzes the oxygenation of various aromatic hydrocarbon substrates. This biotransformation represents the initial event in what is usually a detoxification pathway, which converts lipophilic compounds to water-soluble metabolites. Under some conditions, P4501A1 generates chemically reactive arene oxide intermediates that produce toxic effects by binding covalently to cellular constituents (6). The environmental contaminant TCDD is of particular interest not only because it is the most potent known inducer of P4501A1 but also because it is a poor substrate for such detoxifying enzymes. As a result, TCDD is resistant to metabolic processing and tends to accumulate in tissues over time, a situation that complicates the task of assessing the risk that dioxin poses to human health (2).

The dioxin-responsive *Cyp1A1* gene constitutes an interesting system for analyzing the mechanism by which a chemical signal induces mammalian gene transcription (13, 53). The LMPCR technique allows us to study the *Cyp1A1* gene in its native chromosomal configuration, thereby avoiding potential artifacts that may occur in experimental systems involving reconstituted chromatin or episomes. For example, studies of the steroid hormone-responsive mouse mammary tumor virus (MMTV) promoter reveal that its chromatin structure in an episomal context is not necessarily indicative of its structure in a chromosomal context (1, 28, 36, 40). The results of our DNase I footprinting experiments in nuclei (Fig. 2) agree with and extend those of our previous DMS footprinting studies in intact cells. These findings imply that the preparation of nuclei does not substantially alter protein-DNA interactions at the *Cyp1A1* enhancer-promoter and support the hypothesis that our results accurately reflect the properties of the gene in its native chromosomal setting.

Cyp1A1 induction requires AhR, Arnt, and the dioxin-responsive enhancer and exhibits a graded response to TCDD (20, 53); furthermore, the *Cyp1A1* promoter is silent in the absence of an enhancer (23). Thus, *Cyp1A1* induction involves a transcriptional mechanism that not only switches the gene from an "off" state to an "on" state but also modulates the "on" state in graded fashion. We speculate that other inducible genes which exhibit analogous behavior may utilize a similar mechanism. In contrast, inducible genes that display substantial constitutive (basal) transcription probably use a different mechanism(s), which permits promoter activity in the absence of inducer.

Our previous experiments reveal that when the *Cyp1A1* gene is inactive, the chromatin of the enhancer-promoter region assumes a nucleosomal organization, and general transcription factors fail to bind DNA (34, 54, 55). The repressive effect of nucleosomes on transcription factor binding constitutes a plausible explanation for the inactivity of *Cyp1A1* in uninduced cells (12, 17, 18, 27, 39). The induction of *Cyp1A1* transcription by TCDD is associated with disruption of the nucleosomal organization at the enhancer and with loss of a positioned nucleosome at the promoter, findings that further emphasize a relationship between chromatin structure and *Cyp1A1* gene expression (34).

Our present experiments indicate that induction of transcription is associated with two types of change in *Cyp1A1* enhancer-promoter chromatin: (i) binding of proteins and (ii) increased susceptibility to nuclease digestion. We infer that the binding of AhR/Arnt to the enhancer initiates these changes,

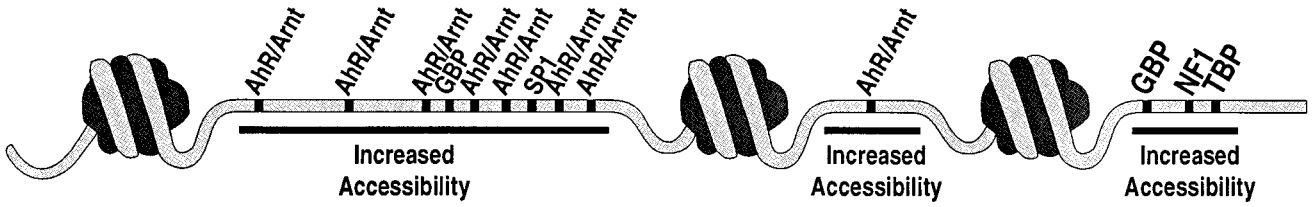

FIG. 7. Summary of TCDD-inducible changes on the *Cyp1A1* regulatory region. Binding sites for transcription factors and regions that exhibit increased nuclease accessibility are indicated. See text for details.

because all are TCDD inducible and AhR/Arnt dependent. The binding of AhR/Arnt to the enhancer is associated with the conversion of about 180 bp of DNA to a nuclease-sensitive configuration; the size of the region suggests that the alteration reflects the disruption of a nucleosome. Therefore, we envision that the increased accessibility of DNA near AhR/Arnt binding sites reflects local nucleosomal disruption. The bending of DNA by AhR/Arnt may help to stabilize the DNA in a nonnucleosomal configuration (8).

A mechanistic explanation for AhR/Arnt's ability to alter chromatin structure must account for our observation that TCDD induces graded increases in the nuclease susceptibility of the *Cyp1A1* enhancer and promoter (Fig. 6). These graded changes parallel the progressive increase in *Cyp1A1* transcription that occurs in response to TCDD (20). One explanation for the graded change is simply that the cell population is heterogeneous with respect to the time course of induction and/or sensitivity to TCDD. Our previous flow cytometric analysis of P4501A1 enzyme induction in individual wild-type cells revealed that the kinetics of induction were similar in all cells. Analyses performed at 3 pM TCDD suggested the existence of some heterogeneity in responsiveness, of undetermined origin; however, at 10 pM and 1 nM TCDD, the cell population responded relatively homogeneously to TCDD (33). These findings imply that heterogeneity in TCDD responsiveness could account for some, but not all, of the graded increase in nuclease susceptibility that TCDD induces at the *Cyp1A1* enhancer and promoter. Therefore, we envision that the graded changes primarily reflect TCDD-induced shifts in an equilibrium that exists between different chromatin structures.

Several forms of "dynamic competition" (3, 12, 49) can account for the changes that occur at the enhancer. One type of mechanism supposes (i) that even when the *Cyp1A1* gene is inactive, each chromatin subunit exists in equilibrium between a nucleosomal state (in which the DNA is "inaccessible") and a nonnucleosomal state (in which the DNA is "accessible") and (ii) that AhR/Arnt can only bind to DNA in the nonnucleosomal state. Raising the concentration of TCDD (which, in turn, increases the concentration of AhR/Arnt) would shift the equilibrium towards the nonnucleosomal, accessible configuration and, as observed, the chromatin in the vicinity of AhR/Arnt binding sites would exhibit enhanced susceptibility to DNase I. This mechanism seems relatively implausible, given the number of histone-DNA and histone-histone interactions that would require spontaneous abrogation for it to occur. A second type of dynamic competition supposes that AhR/Arnt can bind to its recognition sequence in the nucleosome, producing an alteration in structure and stabilizing the DNA in a nonnucleosomal, accessible configuration. Increasing the concentration of TCDD would shift the equilibrium by increasing the concentration of AhR/Arnt at DNA target sites on the enhancer. This mechanism requires that AhR/Arnt be able to overcome the repressive effects of the histones on transcription factor binding. Therefore, AhR/Arnt's ability to invade and disrupt the nucleosome might require additional activities that weaken histone-DNA interactions (such as histone acetylation and/or displacement of histone H1). The process of stabilizing enhancer chromatin in a nonnucleosomal configuration might also require energy and/or "remodeling" factors (3). We note, however, that our analyses reveal no evidence that AhR/Arnt requires additional DNA-binding proteins to alter chromatin structure.

The TCDD-inducible, AhR/Arnt-dependent change in chromatin structure at the *Cyp1A1* promoter must occur by a mechanism different from that at the enhancer, because the promoter contains no AhR/Arnt binding sites. Our analyses of accessibility indicate that the structural change at the promoter is not propagated directly along the chromatin fiber from the enhancer (Fig. 3 and 4). Therefore, AhR/Arnt must target and influence the promoter's nucleoprotein structure from a distance. In addition, the mechanism must allow for a graded change at the promoter in response to TCDD (Fig. 6). Given these constraints, we envision that the binding of AhR/Arnt heteromers to the enhancer allows them to facilitate (via protein-protein interactions) the binding of general transcription factors to promoter DNA. Increasing the concentration of enhancer-bound AhR/Arnt molecules would increase the stability of the transcription complex at the promoter, thereby producing a graded response to TCDD. This hypothesis predicts that enhancer-bound AhR/Arnt molecules contact a protein component(s) of the transcriptional apparatus at the promoter; it is consistent with the observation that both AhR and Arnt exhibit transactivation capability (22, 29, 52).

The mechanism by which AhR/Arnt might facilitate the binding of general transcription factors, such as TBP, to promoter DNA remains to be determined. The inactive promoter may exist in equilibrium between nucleosomal and nonnucleosomal states, and AhR/Arnt may stabilize (directly or via intermediary factors) the binding of general transcription factors to the nonnucleosomal state, thereby shifting the equilibrium towards a nuclease-sensitive configuration. Alternatively, AhR/Arnt may be able to recruit a transcription factor(s) to the promoter and/or alter the properties of a factor(s) so that the protein(s) can actively displace a histone octamer from the promoter. In both instances, TBP-induced DNA bending may help to stabilize the promoter in a nonnucleosomal configuration that is sensitive to nucleases (25, 26). The multiplicity of AhR/Arnt binding sites on the enhancer suggests that several AhR/Arnt molecules simultaneously contact proteins at the promoter, increasing the stability of the transcriptional complex.

We note that AhR/Arnt increases the transcription of naked DNA templates in vitro and of reporter plasmids transiently transfected into cultured cells (24, 50); nucleosomes may not exist on either of these templates. Furthermore, several TCDD-inducible, AhR/Arnt-dependent genes exhibit substantial constitutive activity, findings which imply that their promoters are maintained in an accessible, nonnucleosomal con-

figuration even in the absence of TCDD (11, 38, 41). These observations argue against the idea that AhR/Arnt's primary role in the induction mechanism is to generate an accessible chromatin structure. Instead, we envision that AhR/Arnt recruits additional components of the transcriptional machinery and/or stabilizes the formation of an active transcriptional complex at the promoters of TCDD-responsive genes. Changes in the chromatin structure of the promoter may occur as a result of this primary process.

The promoters for some inducible eukaryotic genes are "preset" in an accessible configuration, and activation of transcription is associated with little change in the structure of promoter chromatin; in contrast, at other promoters, nucleosomes "remodel" to a more accessible configuration during the induction process (49). Our findings indicate that the *Cyp1A1* promoter is in the latter category. Thus, to some extent, the *Cyp1A1* induction mechanism resembles that described for *PHO5*, MMTV, and tyrosine aminotransferase, which also exhibit inducible chromatin remodeling. However, *PHO5* and MMTV differ from *Cyp1A1* in that there is no chromatin region that remains nucleosomal between the promoter and the activator-protein binding site. Therefore, for *PHO5* and MMTV, remodeling appears to result directly from the binding of regulatory proteins in the immediate vicinity of the promoter (10, 45). In the case of tyrosine aminotransferase, the promoter is preset in an accessible configuration, and only the enhancer undergoes remodeling (5). Thus, because AhR/Arnt alters chromatin structure both locally (at the enhancer) and at a distance (at the promoter) without affecting the intervening chromatin, the *Cyp1A1* induction mechanism is different from those described previously.

Our experiments also confirm that substantial differences exist in the ability of transcription factors to bind to inactive chromatin. In the *Cyp1A1* system, some factors (such as AhR/Arnt) are able to access DNA even in the nucleosome and can initiate a transcriptional response. Other factors (such as Sp1 and TBP) cannot interact with nucleosomes and cannot trigger transcription by themselves. The biochemical basis for these functional differences between AhR/Arnt and general transcription factors represents an interesting area for future research.

ACKNOWLEDGMENTS

This research was supported by OIG CA 53887 from the NCI to J.P.W. S.T.O. was supported by fellowship ES05598 from NIEHS.

We thank Margaret Tuggle for secretarial assistance.

REFERENCES

1. **Archer, T. K., E. Zaniewski, M. L. Moyer, and S. K. Nordeen.** 1994. The differential capacity of glucocorticoids and progestins to alter chromatin structure and induce gene expression in human breast cancer cells. Mol. Endocrinol. **8:**1154–1162.
2. **Bailar, J. C., III.** 1991. How dangerous is dioxin? N. Engl. J. Med. **324:**260–262.
3. **Becker, P. B.** 1994. The establishment of active promoters in chromatin. Bioessays **16:**541–547.
4. **Burbach, K. M., A. Poland, and C. A. Bradfield.** 1992. Cloning of the Ah-receptor cDNA reveals a distinctive ligand-activated transcription factor. Proc. Natl. Acad. Sci. USA **89:**8185–8189.
5. **Carr, K. D., and H. Richard-Foy.** 1990. Glucocorticoids locally disrupt an array of positioned nucleosomes on the rat tyrosine aminotransferase promoter in hepatoma cells. Proc. Natl. Acad. Sci. USA **87:**9300–9304.
6. **Conney, A. H.** 1982. Induction of microsomal enzymes by foreign compounds and carcinogenesis by polycyclic aromatic hydrocarbons. Cancer Res. **42:**4875–4917.
7. **Durrin, L. K., and J. P. Whitlock, Jr.** 1989. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin-inducible aryl hydrocarbon receptor-mediated change in *CYP1A1* chromatin structure occurs independently of transcription. Mol. Cell. Biol. **9:**5733–5737.
8. **Elferink, C. J., and J. P. Whitlock, Jr.** 1990. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin-inducible Ah receptor-mediated bending of enhancer DNA. J. Biol. Chem. **265:**5718–5721.
9. **Ema, M., K. Sogawa, N. Watanabe, Y. Chujoh, N. Matsushita, O. Gotoh, Y. Funae, and Y. Fujii-Kuriyama.** 1992. cDNA cloning and structure of mouse putative Ah receptor. Biochem. Biophys. Res. Commun. **184:**246–253.
10. **Fascher, K.-D., J. Schmitz, and W. Hörz.** 1990. Role of *trans*-acting proteins in the generation of active chromatin at the *PHO5* promoter in *S. cerevisiae*. EMBO J. **9:**2523–2528.
11. **Favreau, L. V., and C. B. Pickett.** 1991. Transcriptional regulation of the rat NAD(P)H quinone reductase gene. J. Biol. Chem. **266:**4556–4561.
12. **Felsenfeld, G.** 1992. Chromatin as an essential part of the transcriptional mechanism. Nature (London) **355:**219–224.
13. **Fujii-Kuriyama, Y., H. Imataka, K. Sogawa, K. Yasumoto, and Y. Kikuchi.** 1992. Regulation of *CYP1A1* expression. FASEB J. **6:**706–710.
14. **Garrity, P. A., and B. Wold.** 1992. Effects of different DNA polymerases in ligation-mediated PCR: enhanced genomic sequencing and *in vivo* footprinting. Proc. Natl. Acad. Sci. USA **89:**1021–1025.
15. **Gonzalez, F. J.** 1989. The molecular biology of cytochrome P-450s. Pharmacol. Rev. **40:**243–288.
16. **Gross, D. S., and W. T. Garrard.** 1988. Nuclease hypersensitive sites in chromatin. Annu. Rev. Biochem. **57:**159–197.
17. **Grunstein, M.** 1990. Nucleosomes: regulators of transcription. Trends Genet. **6:**395–400.
18. **Hansen, J. C., and J. Ausio.** 1992. Chromatin dynamics and the modulation of genetic activity. Trends Biochem. Sci. **17:**187–191.
19. **Hoffman, E. C., H. Reyes, F. F. Chu, F. Sander, L. H. Conley, B. A. Brooks, and O. Hankinson.** 1991. Cloning of a factor required for activity of the Ah (dioxin) receptor. Science **252:**954–958.
20. **Israel, D. I., and J. P. Whitlock, Jr.** 1983. Induction of mRNA for cytochrome P_1-450 in wild type and variant mouse hepatoma cells. J. Biol. Chem. **258:**10390–10394.
21. **Israel, D. I., and J. P. Whitlock, Jr.** 1984. Regulation of cytochrome P_1-450 gene transcription by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in wild type and variant mouse hepatoma cells. J. Biol. Chem. **259:**5400–5402.
22. **Jain, S., K. M. Dolwick, J. V. Schmidt, and C. A. Bradfield.** 1994. Potent transactivation domains of the Ah receptor and the Ah receptor nuclear translocator map to their carboxyl termini. J. Biol. Chem. **269:**31518–31524.
23. **Jones, K. W., and J. P. Whitlock, Jr.** 1990. Functional analysis of the transcriptional promoter for the Cyp1A1 gene. Mol. Cell. Biol. **10:**5098–5105.
24. **Jones, P. B. C., D. R. Galeazzi, J. M. Fisher, and J. P. Whitlock, Jr.** 1986. Control of cytochrome P_1-450 gene expression by dioxin. Science **227:**1499–1502.
25. **Kim, J. L., D. B. Nikolov, and S. K. Burley.** 1993. Co-crystal structure of TBP recognizing the minor groove of a TATA element. Nature (London) **365:**520–527.
26. **Kim, Y., J. H. Geiger, S. Hahn, and P. B. Sigler.** 1993. Crystal structure of a yeast TBP/TATA-box complex. Nature (London) **365:**512–520.
27. **Kornberg, R. D., and Y. Lorch.** 1992. Chromatin structure and transcription. Annu. Rev. Cell Biol. **8:**563–587.
28. **Lee, H.-Y., and T. K. Archer.** 1994. Nucleosome-mediated disruption of transcription factor-chromatin initiation complexes at the mouse mammary tumor virus long terminal repeat in vivo. Mol. Cell. Biol. **14:**32–41.
29. **Li, H., L. Dong, and J. P. Whitlock, Jr.** 1994. Transcriptional activation function of the mouse Ah receptor nuclear translocator. J. Biol. Chem. **269:**28098–28105.
30. **Matsushita, N., K. Sogawa, M. Ema, A. Yoshida, and Y. Fujii-Kuriyama.** 1993. A factor binding to the xenobiotic responsive element (XRE) of P-450IA1 gene consists of at least two helix-loop-helix proteins, Ah receptor and Arnt. J. Biol. Chem. **268:**21002–21006.
31. **McGhee, J. D., and G. Felsenfeld.** 1979. Reaction of nucleosomal DNA with dimethyl sulfate. Proc. Natl. Acad. Sci. USA **76:**2133–2137.
32. **McPherson, C. E., E.-Y. Shim, D. S. Friedman, and K. S. Zaret.** 1993. An active tissue-specific enhancer and bound transcription factors existing in a precisely positioned nucleosomal array. Cell **75:**387–398.
33. **Miller, A. G., and J. P. Whitlock, Jr.** 1982. Heterogeneity in the rate of benzo[*a*]pyrene metabolism in single cells: quantitation using flow cytometry. Mol. Cell. Biol. **2:**625–632.
34. **Morgan, J. E., and J. P. Whitlock, Jr.** 1992. Transcription-dependent and transcription-independent nucleosome disruption induced by dioxin. Proc. Natl. Acad. Sci. USA **89:**11622–11626.
35. **Mueller, P. R., and B. Wold.** 1989. In vivo footprinting of a muscle specific enhancer by ligation mediated PCR. Science **246:**780–786.
36. **Mymryk, J. S., D. Berard, G. L. Hager, and T. K. Archer.** 1995. Mouse mammary tumor virus chromatin in human breast cancer cells is constitutively hypersensitive and exhibits steroid hormone-independent loading of transcription factors in vivo. Mol. Cell. Biol. **15:**26–34.
37. **Nebert, D. W., and J. E. Jones.** 1989. Regulation of the mammalian cytochrome P_1-450 (CYP1A1) gene. Int. J. Biochem. **21:**243–252.
38. **Okino, S. T., U. R. Pendurthi, and R. H. Tukey.** 1992. Phorbol esters inhibit the dioxin receptor-mediated transcriptional activation of the mouse Cyp1a1 and Cyp1a2 genes by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. J. Biol. Chem. **267:**6991–6998.

39. **Paranjape, S. M., R. T. Kamakama, and J. T. Kadonaga.** 1994. Role of chromatin structure in the regulation of transcription by RNA polymerase II. Annu. Rev. Biochem. **63:**265–297.
40. **Pennie, W. D., G. L. Hager, and C. L. Smith.** 1995. Nucleoprotein structure influences the response of the mouse mammary tumor virus promoter to activation of the cyclic AMP signalling pathway. Mol. Cell. Biol. **15:**2125–2134.
41. **Pimental, R. A., B. Liang, G. K. Yee, A. Wilhelmsson, L. Poellinger, and K. E. Paulson.** 1993. Dioxin receptor and C/EBP regulate the function of the glutathione *S*-transferase Ya gene xenobiotic response element. Mol. Cell. Biol. **13:**4365–4373.
42. **Poland, A., and J. C. Knudson.** 1982. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin and related aromatic hydrocarbons: examination of the mechanism of toxicity. Annu. Rev. Pharmacol. Toxicol. **22:**517–554.
43. **Probst, M. R., S. Reisz-Porszasz, V. Agbunag, M. S. Ong, and O. Hankinson.** 1993. Role of the aryl hydrocarbon receptor nuclear translocator protein in aryl hydrocarbon (dioxin) receptor action. Mol. Pharmacol. **44:**511–518.
44. **Reyes, H., S. Reiz-Porszasz, and O. Hankinson.** 1992. Identification of the Ah receptor nuclear translocator protein (Arnt) as a component of the DNA binding form of the Ah receptor. Science **256:**1193–1195.
45. **Richard-Foy, H., and G. Hager.** 1987. Sequence-specific positioning of nucleosomes over the steroid-inducible MMTV promoter. EMBO J. **6:**2321–2328.
46. **Safe, S. H.** 1986. Comparative toxicology and mechanism of action of polychlorinated dibenzo-*p*-dioxins and dibenzofurans. Annu. Rev. Pharmacol. Toxicol. **26:**371–399.
47. **Swanson, H. I., and C. A. Bradfield.** 1993. The AH-receptor: genetics, structure and function. Pharmacogenetics **3:**213–230.
48. **Tanguay, R. L., G. D. Pfeifer, and A. D. Riggs.** 1990. PCR-aided DNase I footprinting of single copy gene sequences in permeabilized cells. Nucleic Acids Res. **18:**5902.
49. **Wallrath, L. L., Q. Lu, H. Granok, and S. C. R. Elgin.** 1994. Architectural variations of inducible eukaryotic promoters: preset and remodeling chromatin structures. Bioessays **16:**165–170.
50. **Wen, L. P., N. Koeiman, and J. P. Whitlock, Jr.** 1990. Dioxin-inducible, Ah receptor-dependent transcription *in vitro*. Proc. Natl. Acad. Sci. USA **87:**8545–8549.
51. **Whitelaw, M., I. Pongratz, A. Wilhelmsson, J.-Å. Gustafsson, and L. Poellinger.** 1993. Ligand-dependent recruitment of the Arnt coregulator determines DNA recognition by the dioxin receptor. Mol. Cell. Biol. **13:**2504–2514.
52. **Whitelaw, M. L., J.-Å. Gustafsson, and L. Poellinger.** 1994. Identification of transactivation and repression functions of the dioxin receptor and its basic helix-loop-helix/PAS partner factor Arnt: inducible versus constitutive modes of regulation. Mol. Cell. Biol. **14:**8343–8355.
53. **Whitlock, J. P., Jr.** 1993. Mechanistic aspects of dioxin action. Chem. Res. Toxicol. **6:**754–763.
54. **Wu, L., and J. P. Whitlock, Jr.** 1992. Mechanism of dioxin action: Ah receptor-mediated increase in promoter accessibility *in vivo*. Proc. Natl. Acad. Sci. USA **89:**4811–4815.
55. **Wu, L., and J. P. Whitlock, Jr.** 1993. Mechanism of dioxin action: receptor-enhancer interactions in intact cells. Nucleic Acids Res. **21:**119–125.